

A defect in the yeast plasma membrane urea transporter *Dur3p* is complemented by *CpNIP1*, a Nod26-like protein from zucchini (*Cucurbita pepo* L.), and by *Arabidopsis thaliana* δ -TIP or γ -TIP

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Abstract *Dur3* encodes the yeast plasma membrane urea transporter and *Adur3* mutants are unable to grow on media containing low concentrations of urea as sole nitrogen source. Complementation of the *Adur3* mutant line with expression libraries generated from whole *Arabidopsis thaliana* seedlings or from zucchini (*Cucurbita pepo* L.) vascular tissue yielded numerous lines that had regained the capacity to grow on low urea as sole nitrogen source. Analysis of several of these yeast lines revealed that the *Adur3* mutation was complemented either by δ -TIP (TIP = tonoplast intrinsic protein) or γ -TIP from *Arabidopsis* or by *CpNIP1*, a new NOD26-like protein from zucchini. δ -TIP (At3g16240) and γ -TIP (At2g36830) had previously been characterized as proteins facilitating the transport of water across the tonoplast membrane, and Nod26-like proteins were characterized as glycerol transporters. So far, transport of urea has not been described for any of the proteins described in this paper. Further analyses support this function of TIPs and nodulin 26-like intrinsic proteins in urea transport.

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1. Introduction

The MIP (major intrinsic protein) family of proteins includes members found most likely in all cellular organisms [1,2]. This gene family is particularly diverse in higher plants, where more than 30 homologous genes have been identified in *Arabidopsis* [3] and a similar number of independent expressed sequence tags have been found in maize [4]. These large families can be divided into four phylogenetic subgroups [2,3]: the plasma membrane intrinsic proteins (PIPs), the tonoplast intrinsic proteins (TIPs), the nodulin 26-like intrinsic proteins (NIPs) and the small basic intrinsic proteins (SIPs).

Typically, analyses of MIP functions were performed by injection of cRNAs into *Xenopus laevis* oocytes, where volume changes and cell rupture under hypo-osmotic conditions demonstrated the water transport capacity of these proteins [5–7]. Injections of cRNA from *Arabidopsis* γ -TIP increased the water permeability of *Xenopus* plasma membranes 6–15-fold

over control values [6,7], and similar analyses were also performed for δ -TIP [7] and α -TIP [8].

Other MIP family members, such as the GlpF protein from *Escherichia coli*, catalyze the passage of small polyols, such as glycerol, xylitol or ribitol, but not of water [9]. Moreover, it has been shown for the mammalian aquaporin-3 (AQP3) that in addition to being a water channel it can also transport small solutes, such as glycerol or urea [10,11]. The amino acids involved in these varying specificities were identified, when mutations of a specific set of amino acids in a water channel resulted in a switch to a glycerol-specific protein [12].

Structural analyses of aquaporin-1 (AQP1), a water channel from red blood cells [13], revealed that AQPs form tetramers in the membrane [14–16] and that each subunit forms an independent water-conducting unit [17]. The three-dimensional structures of AQP1 [18] and of the glycerol-specific GlpF from *E. coli* [19] identified the differences in the selectivity filters within these monomers. However, inhibitor studies on the aquaglyceroporin AQP3 [20] as well as the three-dimensional structure obtained for GlpF [19] suggested that the center of the tetrameric proteins might form an additional channel of its own.

For plant MIPs the transport of glycerol in addition to water was first published for GmNOD26 from *Glycine max* [21], and later also for NtAQP1 [22] and for NtTIPa [23], both from tobacco. Whereas these data were obtained from cRNA-injected *Xenopus* oocytes, glycerol transport for AtNLM1 and AtNLM2, two NIPs from *Arabidopsis*, was determined after cDNA expression in baker's yeast [24]. Interestingly, only for NtTIPa uptake of urea in addition to glycerol was observed [23]. This was explained by differences between NtTIPa and all other, previously described TIPs.

Here we show by complementation of a urea transport-deficient line of baker's yeast and by additional transport and growth analyses that *Arabidopsis* γ -TIP and δ -TIP as well as the *CpNIP1* protein from zucchini can complement this defect. Moreover, we show for the first time that the defect in a yeast plasma membrane transporter can be complemented by plant cDNAs encoding vacuolar proteins.

2. Materials and methods

2.1. Strains and growth conditions

The *DUR3* wild type strain FY1679-13c (MATa, *ura3-52*, *his3D200*, *DUR3*) was obtained from the EUROSCARF strain collection (Institute for Microbiology, Johann Wolfgang Goethe University, Frankfurt, Germany). All cloning steps in *E. coli* were performed

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in strain DH5 α [25]. Zucchini plants (*C. pepo* L.) used for the isolation of leaf veins were grown under ambient conditions in the field.

2.2. Disruption of the *DUR3* gene

For disruption of the yeast *DUR3* gene a linear DNA fragment containing the *Schizosaccharomyces pombe* *HIS5* gene and short flanking *DUR3* sequences was generated by polymerase chain reaction (PCR) using the oligonucleotides ScDur3g-50f/his3 (5'-TTG GGT TCA TCC AAG AAA AAG TAA TAA CAT AAA TTT GTC ATT GTT ATA GTC GTA CGC TGC AGG TCG AC-3') and ScDur3g+2257r/his3 (5'-AAA AAT TAT TAA TGA GGT TGA TGA GTG GAT AGA ATA TTA TAT TTT GAA AGA TCG ATG AAT TCG AGC TCG-3') and the plasmid pFA6a-HIS3MX6 [26] as template (regions of the primer sequences binding to the *S. pombe* *HIS5* gene are in italics). The resulting PCR fragment was used for the transformation [27] of *Saccharomyces cerevisiae* cells (FY1679-13c) and the disruption of the *DUR3* gene. Cells carrying the disruption were able to grow in the absence of histidine in the medium. The *dur3* deletion was confirmed by sequencing. The resulting strain was named FY1679:: Δ dur3.

2.3. Construction of the zucchini library

Poly(A) mRNA was isolated from excised veins of zucchini leaves (*C. pepo* L.) using standard protocols and transcribed into double-stranded cDNA with a cDNA synthesis kit from Stratagene (La Jolla, CA, USA). cDNAs were ligated in an oriented manner into the yeast/*E. coli* shuttle/expression vector pMW3, which represents a modification of the plasmid pFL61 [28]. For the construction of pMW3, unique *EcoRI* and *XhoI* cloning sites were inserted between the *NotI* sites of pFL61 using the annealed oligonucleotides EcoXho-Linker1 (5'-GGC CGG AAG AAT TCA AGC TTG TAA AAG ACT CGA GC-3') and EcoXho-Linker2b (5'-GGC CGC TCG AGT CTT TTA GAA GCT TGA ATT CTT GC-3').

2.4. Complementation of the yeast *dur3* mutation with plant cDNA libraries

For complementation of the Δ dur3 deletion, yeast cells were transformed with expression libraries from *Arabidopsis* [28] or zucchini (this paper) and double-selected for growth in the absence of uracil with 2 mM urea as sole nitrogen source.

2.5. Transport tests in baker's yeast

Transport of [14 C]urea was measured in baker's yeast as previously described [29].

2.6. Yeast growth analyses

Growth of yeast cells was analyzed on synthetic dextrose medium (2% D-glucose supplemented with vitamins) with varying concentrations of urea, in the presence or absence of sorbitol, glycerol or KCl.

3. Results

3.1. Complementation of the yeast *dur3* mutant

Urea is frequently used as a plant fertilizer [30] suggesting that plants might be able to import urea from the soil. Clearly, urea seems to interfere with the uptake of other nitrogen sources [31], but little is known about the molecular basis of urea uptake in higher plants. For lower plants, such as *Chara australis* [32] or the marine diatom *Phaeodactylum tricornutum* [33], urea uptake has been studied using electrophysiological techniques and it has been suggested that urea is imported via a Na⁺ symport mechanism.

For the identification of possible plant urea transporters we generated a yeast mutant (FY1679:: Δ dur3) carrying a deletion in its endogenous urea transporter gene *DUR3*. FY1679:: Δ dur3 cells were unable to grow on low concentrations (2 mM) of urea as sole nitrogen source (Fig. 1, first row). This mutant line was transformed with two different plant cDNA libraries that had been generated in the yeast expression vector pFL61 [28]. The libraries had been prepared either from complete seedlings of *Arabidopsis thaliana* [28] or from excised veins of zucchini leaves (*C. pepo* L.).

About 50 000 FY1679:: Δ dur3 transformants of the *Arabidopsis* library and about 100 000 transformants of the zucchini library were grown on Petri plates containing minimal me-

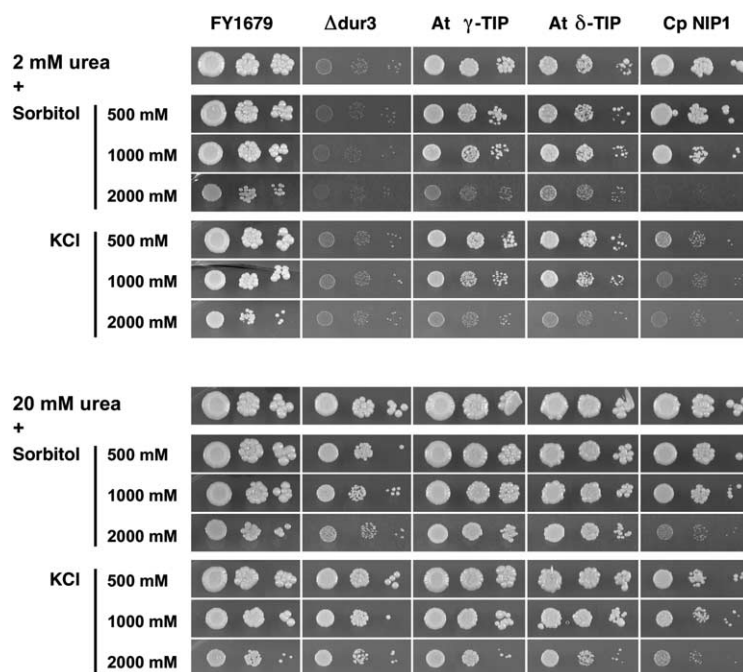


Fig. 1. Growth analysis of *DUR3* wild type cells (FY1679), of the isogenic FY1679:: Δ dur3 mutant line (Δ dur3), and of three FY1679:: Δ dur3 lines complemented with the indicated plant MIP cDNAs (At γ -TIP, At δ -TIP, CpNIP1). Cells (three different dilutions) were spotted on Petri plates containing 2 mM urea (upper panel) or 20 mM urea (lower panel) as sole nitrogen source. Plates either contained no extra osmolyte (first row of each panel) or were supplemented with the indicated concentrations of sorbitol or KCl. All plates were incubated at 29°C for the same time.

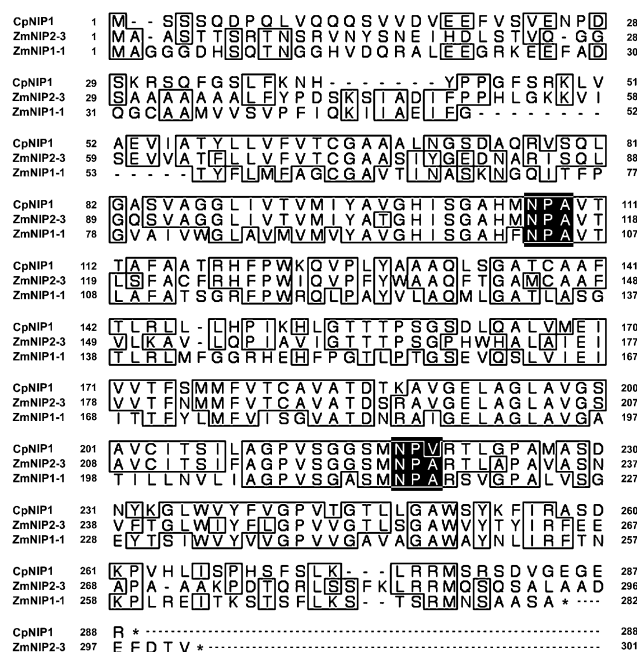


Fig. 2. Alignment of CpNIP1, ZmNIP1;1 and ZmNIP2;3 protein sequences. Amino acids identical in CpNIP1 and the other proteins are boxed. 'NPA' boxes, which are typical for plant MIPs, are highlighted in black. The cDNA sequence of CpNIP1 is deposited in the EMBL data library (accession number: AJ544830).

dium supplemented with 2 mM urea as sole nitrogen source. After 4 days of incubation at 29°C, 17 colonies (=0.03%) resulting from the *Arabidopsis* complementation and 480 colonies (=0.5%) resulting from the zucchini complementation were identified on plates with 2 mM urea.

3.2. Identification of TIPs and NIPs

Restriction analyses of the plasmids from 12 randomly picked colonies of each complementation revealed similar insert sizes for all *Arabidopsis* clones and identical inserts for all zucchini clones. Sequencing of several inserts revealed that all inserts represented members of the higher plant MIP gene family. cDNAs obtained from the *Arabidopsis* library corresponded to δ -TIP or γ -TIP, two proteins previously characterized as vacuolar water channels [6,7] and cDNAs obtained from the zucchini library encoded a new NOD26-like protein. This new gene was named CpNIP1 (*C. pepo* NOD26-like intrinsic protein 1). Genes encoding NOD26-like proteins have previously been described in other plants, such as in *Glycine max* (*GmNOD26* [34]), in *Arabidopsis* (*NLM1* and *NLM2* [24]) or in corn (*ZmNIP1;1* or *ZmNIP2;3* [4]). An alignment of the CpNIP1 protein sequence with the sequences of its nearest homologs, ZmNIP1;1 and ZmNIP2;3, is presented in Fig. 2 confirming that CpNIP1 is a member of the NIP subgroup of plant MIPs. CpNIP1 encodes a protein of 288 amino acids with a calculated molecular mass of 30.45 kDa and an isoelectric point of 8.9. CpNIP1 shares 51% and 68% identity at the protein level with ZmNIP1;1 or ZIP2;3 [4] and only 35% or 38% identity with At γ -TIP [6] or NtTIPa [23]. It shows the typical 'NPA' boxes found in all MIPs (highlighted in Fig. 2), but the second box is slightly modified to 'NPV', similar to the situation found in *Arabidopsis* NIPs that contain 'NPG' at this position [24].

From all previously identified plant NIP genes only the cDNAs of the *Arabidopsis* NLM genes were analyzed by heterologous expression in yeast [24]. These analyses had shown that AtNML1 and AtNML2 catalyze the transport of glycerol into the transformed yeast cells.

FY1679:: Δ dur3 cells expressing At γ -TIP, At δ -TIP or CpNIP1 were named YFW1, YFW2 and YFW3, respectively. These three cell lines were plated together with the isogenic wild type and mutant lines (FY1679-13c and FY1679:: Δ dur3) on Petri plates containing 2 mM or 20 mM urea as sole nitrogen source. Comparison of the cell growth under these conditions revealed that the complementation phenotype was visible only on medium with 2 mM urea (Fig. 1, first row of upper panel). On plates with 20 mM urea, FY1679-13c wild type cells, the three complementants and even the FY1679:: Δ dur3 mutant line grew almost equally well indicating that this concentration is high enough to allow sufficient diffusion of urea into the mutant cells (Fig. 1, first row of lower panel). This is additional indirect evidence that the enhanced growth rates of the complemented strains on 2 mM urea can only result from enhanced urea influx into these yeast lines.

3.3. Complemented cells show enhanced sensitivity to high solute concentrations

Yeast strains with plant water channels in their plasma membrane might show an enhanced sensitivity to osmotic stress, if these channels mediate an unregulated water loss at

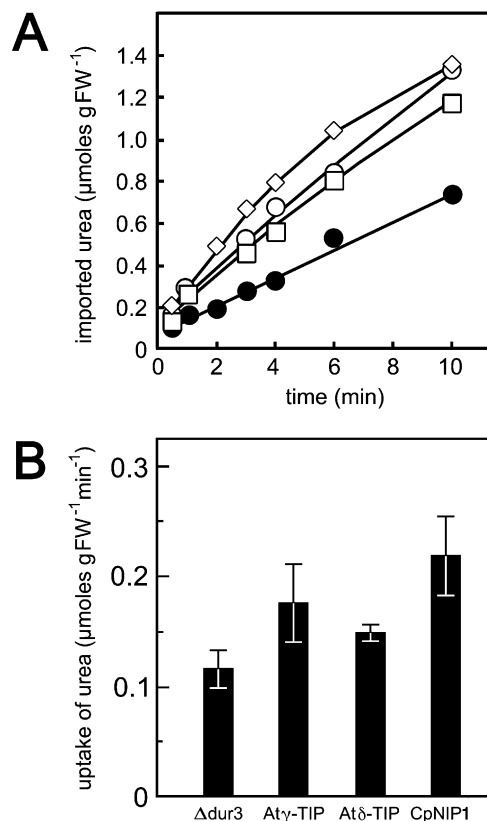


Fig. 3. A: Typical analysis of [14 C]urea uptake by the Δ dur3 mutant and by three different, complemented cell lines. The initial outside concentration was 1 mM in all analyses (●: FY1679:: Δ dur3 cells; ○: FFW1 cells (At γ -TIP); □: FFW2 cells (At δ -TIP); ◇: FFW3 cells (CpNIP1)). B: Statistical analysis (mean \pm S.E.M.) of five independent data sets performed as in A.

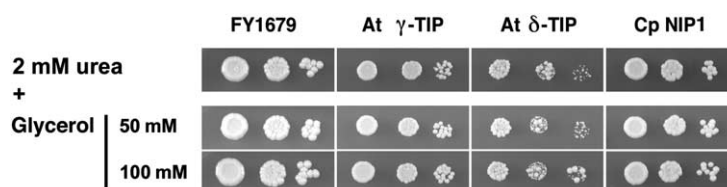


Fig. 4. Growth analysis of FY1679:: Δ dur3 cells and of the isogenic complementants on urea plates with and without glycerol. Growth of FY1679:: Δ dur3 mutant cells and of complemented Fwy1, Fwy2 and Fwy3 cells (expressing *At γ -TIP*, *At δ -TIP* or *CpNIP1*) was analyzed on Petri plates containing 2 mM urea as sole nitrogen source and 2% glucose as carbon source (top row). Different concentrations of glycerol (50 mM or 100 mM) were added to study a potentially inhibiting effect of glycerol on urea utilization.

high extracellular solute concentrations. We tested this possibility and analyzed colony growth on Petri plates in the presence of increasing concentrations (0 mM, 500 mM, 1 M, 2 M) of sorbitol or KCl, both on 2 mM and on 20 mM urea. The strongest sensitivity to high solute concentrations was observed for the *CpNIP1*-expressing cells (FWY3). On plates with 2 mM urea, growth of Fwy3 cells was suppressed almost completely in the presence of 2 M sorbitol and strongly affected already at 500 mM KCl (Fig. 1, upper panel). Under these conditions, also Fwy1 (expressing *At γ -TIP*) and Fwy2 (expressing *At δ -TIP*) were slightly more sensitive than the wild type strain FY1679-13c, but clearly less sensitive than Fwy3.

The increased sensitivity of Fwy3 cells was also observed, although less pronounced, if the experiment was performed in the presence of 20 mM urea (Fig. 1, lower panel). Growth was inhibited almost completely by 2 M sorbitol and strongly reduced by all concentrations of KCl.

The presented results demonstrate that *CpNIP1* mediates the transmembrane passage of urea and water, because its cDNA can complement the defect in *DUR3* and because *CpNIP1* expression enhances the sensitivity of yeast cells to high extracellular solute concentrations.

3.4. Transport of radiolabelled urea and competition with glycerol

To further support the results from the growth data, uptake of urea was analyzed directly in FY1679:: Δ dur3 mutant cells and in Fwy1, Fwy2 and Fwy3 complementants using 14 C-labelled substrate. Uptake rates for [14 C]urea were determined at initial outside concentrations of 1 mM (Fig. 3) and 5 mM (not shown) over various lengths of time. A typical transport test is shown in Fig. 3. Reproducibly, the rates determined for FY1679:: Δ dur3 mutant cells (closed circles in Fig. 3A) were smaller than the rates determined for the three complemented lines. The analyses shown in Fig. 3B represent mean values of five experiments and demonstrate that the [14 C]urea transport rates are 30–40% increased in the *TIP*-expressing lines Fwy1 and Fwy2, and about 80% increased in the *CpNIP1*-expressing line Fwy3. This is in perfect agreement with the data presented in Fig. 1, where Fwy3 shows the best growth on 2 mM urea of all three complemented lines (upper panel).

It has been reported [24] that the *A. thaliana* NLM1 and NLM2 proteins possess a glycerol permease activity, when expressed in baker's yeast. Therefore, we tested the effect of excess glycerol on the transport of [14 C]urea in the FY1679:: Δ dur3 mutant cells and in the *CpNIP1*-expressing line Fwy3. We were not able to detect any inhibition of urea transport in the Fwy3 cells (data not shown). Moreover, we tested if the presence of different concentrations of glycerol in the growth

medium (0 mM, 50 mM, 100 mM) interferes with the ability of the *CpNIP1*-expressing Fwy3 cells to grow on 2 mM urea as sole nitrogen source. Fig. 4 shows that neither a 25-fold nor a 50-fold excess of glycerol has a negative effect on the ability of Fwy3 cells to use low concentrations of urea, supporting the above-mentioned result that glycerol is not transported by *CpNIP1*.

4. Discussion

4.1. *CpNIP1* mediates the transport of urea and water, but not of glycerol

The data presented in this paper demonstrate that *CpNIP1*, a new higher plant NOD26-like protein from zucchini (*C. pepo* L.), catalyzes the transmembrane flow of urea and water. This is supported by the identification of numerous *CpNIP1*-expressing yeast lines in complementation analyses of a Δ dur3 mutant of *S. cerevisiae* that was transformed with an expression library from enriched zucchini vascular tissue (Fig. 1). In wild type yeast cells, Dur3p catalyzes the transport of urea across the yeast plasma membrane [35]. Urea transport by *CpNIP1* is also supported by the increased uptake rates ob-

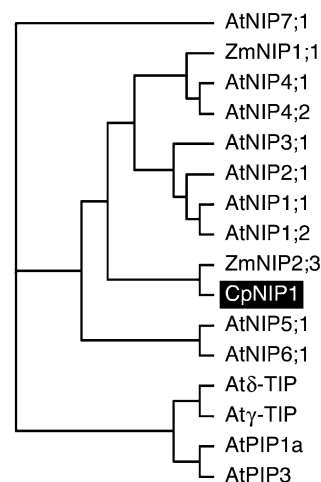


Fig. 5. Cladogram of *CpNIP1*, all nine members of the *Arabidopsis* *AtNIP* family [3], two members of the *Arabidopsis* *TIP* family, two members of the *Arabidopsis* *PIP* family and two *NIP* sequences from corn (MIP numbers of the corresponding *Arabidopsis* genes: *AtNIP1;1*: At4g18910; *AtNIP1;2*: AtNIP1;2; *AtNIP2;1*: At2g34390; *AtNIP3;1*: At1g31885; *AtNIP4;1*: At5g37810; *AtNIP4;2*: At5g37820; *AtNIP5;1*: At4g10380; *AtNIP6;1*: At1g80760; *AtNIP7;1*: At3g06100; *At δ -TIP*: At3g16240; *At γ -TIP*: At2g36830; *AtPIP1a*: At3g61430; *AtPIP3*: At2g16835. GenBank accession numbers for all other genes: *ZmNIP1;1*: AF326483; *ZmNIP2;3*: AF342810; *CpNIP1*: AJ544830). The cladogram was generated using the programs ClustalX [36] and TREEVIEW [37].

served with radiolabelled [^{14}C]urea in *CpNIP1*-expressing yeast cells (Fig. 3). This function of a NOD26-like protein is new, because neither NOD26 from soybean symbiosomes [21] nor the analyzed NOD26-like proteins from *Arabidopsis* [24] catalyze the transport of urea. These proteins are permeable to glycerol [21,24] and GmNOD26 can also catalyze the transmembrane passage of formamide [24].

The presented data suggest that *CpNIP1* is also a transmembrane water channel, because *CpNIP1* expression in baker's yeast results in an enhanced sensitivity to high extracellular solute concentrations (Fig. 1). Such a dual function in water and solute transport was also described for GmNOD26 [21] and other MIPs.

Finally, our analyses suggest that *CpNIP1* cannot catalyze the transmembrane flux of glycerol for two reasons. Firstly, no inhibition of [^{14}C]urea transport by excess glycerol was observed in FWY3 cells (data not shown). Secondly, a 50-fold excess of glycerol in the growth medium did not reduce the capacity of FWY3 cells to utilize low concentrations of urea (Fig. 4).

Of the large MIP family identified in *Arabidopsis* nine members were assigned to the cluster of Nod26-like proteins and named AtNIPs [3]. Fig. 5 shows that the *CpNIP1* gene from zucchini clearly belongs to this cluster of AtNIPs, but the orthologous gene from *Arabidopsis* cannot be identified from this cladogram. A good candidate is AtNIP6;1, which is quite closely related to *CpNIP1* and which is the only *Arabidopsis* NIP that has the identical NPA...NPV signature that is found in *CpNIP1* (Fig. 2). ZmNIP2;3 from corn, although having a NPA...NPA signature (Fig. 2), is more closely related to *CpNIP1* than all *Arabidopsis* MIPs.

Interestingly, no *Arabidopsis* or corn PIPs were identified in our complementation analyses, suggesting that these transporters are unable to mediate the transmembrane flux of urea. In fact, yeast cells transformed with an expression vector (pFL61 [28]) harboring the PCR-generated open reading frames of *AtPIP1a* (At3g61430) or *AtPIP3* (At2g16835) did not grow on medium with 2 mM urea as sole nitrogen source (data not shown).

4.2. *CpNIP1* is prominent in zucchini leaf veins

The number of yeast cells harboring a complementing plasmid after transformation of the FY1679:: Δdur3 mutant with the zucchini library was unexpectedly high. About 0.5% of the transformed cells were able to grow on low urea and 12 randomly picked colonies contained the identical insert that was characterized as *CpNIP1*. In contrast, only 0.03% of the yeast cells transformed with the *Arabidopsis* library were able to utilize low urea, and the inserts of all analyzed clones (40% of all clones) were characterized as γ -TIP and δ -TIP.

It cannot be excluded that a small number of cDNAs different from *CpNIP1* might be found amongst the large number of zucchini clones and it cannot be excluded that analyses of a larger number of *Arabidopsis* clones might identify other MIP genes. However, the large number of *CpNIP1* transformants obtained with the library from zucchini leaf veins suggests a high expression level for the corresponding gene in this tissue. If this expression is specific for the vascular tissue or if similar levels can be found in other parts of the leaf or of the entire zucchini plant will need to be analyzed in additional experiments, including studies on the organ, tissue and cell specificity of *CpNIP1* expression.

4.3. *Arabidopsis* vacuolar water channels γ -TIP and δ -TIP catalyze the flow of urea

In contrast to *CpNIP1*, the function of γ -TIP and δ -TIP has been well characterized and both proteins had previously been described as vacuolar water channels [6,7]. Our results on γ -TIP and δ -TIP are new and interesting for several reasons. Firstly, our data clearly show that these two proteins can catalyze the transmembrane flux of urea, because they complement the Δdur3 mutation (Fig. 1), and because expression of their cDNAs confers the capacity to enhanced [^{14}C]urea transport (Fig. 3). So far, transport of urea has only been described for NtTIPa from tobacco [23], and γ -TIP and δ -TIP were thought to be specific for water. However, detailed analyses of the permeability of tobacco tonoplast vesicles [23] revealed that these membranes are highly permeable to urea. Inhibitor studies suggested that this permeability is mediated by proteinaceous pores that are sensitive to copper and mercuric chloride [23]. Eventually, these studies led to the cloning of NtTIPa, which turned out to catalyze the transport of water, urea and glycerol [23]. These studies also showed that, compared to plasma membrane vesicles, tonoplast vesicles exhibited a 35–70-fold higher permeability to urea. This may explain why PIPs have not been found in our complementation analyses.

Secondly, our data show that it is possible to study the function of higher plant vacuolar proteins in intact yeast cells, because at least part of the recombinant protein may be targeted to the plasma membrane. The function of this protein can then be studied directly in transport analyses as shown in Fig. 3. This demonstrates that complementation of yeast cells may also be used as a screening system for the identification and functional characterization of vacuolar transport systems. This may not apply to every vacuolar protein and it may depend on the quality and intensity of the expression in yeast. Nevertheless, the identification of γ -TIP and δ -TIP in yeast plasma membranes shows that this is a possible way to go. Our data also show that the identification of a recombinant membrane protein in the yeast plasma membrane does not necessarily allow a prediction on the localization of this protein in its original system.

Our results on the new *CpNIP1* protein and on the previously described *Arabidopsis* TIPs support the idea that many (or possibly all) plant aquaporins transport water and small solutes in addition, just as their homologous counterparts in bacteria or animals. However, it will need to be studied in further analyses, whether the transport of urea, which was analyzed in this paper, is of physiological significance in planta.

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